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Effects of Glucocorticoids and Mineralocorticoids on Proliferation and Maturation of Human Peripheral Blood Stem Cells

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It has been shown that hematopoietic progenitors can be expanded ex vivo in the presence of various cytokine combinations. Glucocorticoids (GC) are involved in the self-renewal of erythroid progenitors in chicken. To see whether GC have a similar effect on hematopoiesis in humans, CD34$^+$ peripheral blood stem cells were cultured in serum free medium in the presence of a GC, triamcinolone acetonide. However, our results demonstrate an inhibition of both erythroid and granulocyte-macrophage (GM) proliferation and a modification of erythroid colony morphology. Furthermore, RU38486 (Mifepristone), a potent GC antagonist, was unable to reverse the inhibitory effect of triamcinolone acetonide. We also identified and characterized another steroid subfamily, the mineralocorticoid (MC) subfamily, in human PB CD34$^+$ cells. The MC, aldosterone, significantly enhanced GM colony formation and diminished the erythroid colony number. Neither of effects were inhibited by ZK91587, an antagonist specific to the MC receptor (MCR). In contrast, ZK91587 reversed the stimulatory effect of deoxycorticosterone on GM colony formation. Cytoplasmic staining for MCR was observed in CD34$^+$ cells incubated with a polyclonal antiserum raised against human MCR. To our knowledge, this is the first demonstration of the presence of MCR in human PB CD34$^+$ cells. Am. J. Hematol. 62: 65–73, 1999. © 1999 Wiley-Liss, Inc.

Key words: human; peripheral blood; glucocorticoid; mineralocorticoid; mineralocorticoid receptor

INTRODUCTION

Lipophilic hormones including steroids influence the development, differentiation and homeostasis of a large number of cells. Steroids bind and activate intracellular receptors, which are direct modulators of transcription [1,2]. The receptor recognizes the cognate hormone response element (HRE) of the proximal promoter by means of two zinc fingers [3–8]. The mineralocorticoid (MC) receptor (MCR) is the largest member in the steroid subfamily, which also contains receptors for androgens, progestins and glucocorticoids (GC), and which recognize an identical HRE with the exception of members of the estrogen receptor subfamily which recognize a different HRE [6,7]. MCR is a 8-9S heterooligomeric complex that includes the 90 kDa heat shock protein (hsp90) [9]. MC and GC hormones elicit distinct physiological responses, yet the MCR and GC receptor (GCR) bind to and activate transcription similarly from a consensus simple HRE. Glucocorticoids are steroid hormones that exert a multitude of biological effects through the action of a specific receptor protein [10]. The transcriptional response to GC can be blocked by potent antagonist like RU38486 (Mifepristone). GC are involved in the control of various physiological processes such as inflammation [11,12] and are widely used as antiinflammatory, immunosuppressive, and antiallergic agents. GC gain access to the cell mainly by the process of simple diffusion through the plasma membrane. In the absence of ligand, GCR exists as a large heterodimeric

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complex comprising a single GCR molecule, a dimer of hsp90, as well as two other proteins hsp56 and hsp70. Upon binding ligand, the GCR becomes activated and acquired the ability to bind DNA.

Hematopoiesis in vivo is dependent upon the interaction of hematopoietic stem cells (HSC) with a complex microenvironment of which stromal protoglycans are important functional components. Certain bone marrow stromal cell lines provide, if GC are supplied, a microenvironment that supports hematopoiesis in vitro [13]. GCR have been identified as key regulators of an important developmental decision in hematopoiesis [14]. Progenitor cells sometimes generate and maintain a certain number of immature cells, which proliferate without apparent differentiation. Such a mechanism exists not only in pluripotent progenitor cells [15] but also in certain committed progenitors [16]. To maintain a balanced composition of hematopoietic cells, progenitor cells have to regulate proliferation and differentiation. Hematopoietic progenitor cells generate a large number of differentiated progenitors [17]. GC inhibit the formation of murine granulocyte-macrophage (GM) colonies [18], but enhance the formation of erythroid colonies in vitro [19–21]. In the presence of GC, lower concentrations of Epo are required to induce maximal erythroid cell proliferation [22]. In vivo, GC can restore normal erythropoiesis in pediatric congenital aplastic anemia [23]. Treatment of nonanemic patients with prednisone, the synthetic GCR ligand, results in increased erythropoiesis [24].

These findings suggest that the ligand-activated GCR enhances the proliferative capacity of erythroid progenitors. Furthermore, there is evidence that the GCR is required for erythroid progenitor self-renewal from bone marrow chicken cells by inducing differentiation arrest of primary erythroid progenitors [25]. The partial GCR antagonist RU38486, which can induce nuclear translocation of the GCR, acts as a pure antagonist in erythroid cells. No similar study was made in vitro on $CD34^+$ peripheral blood HSC collected from human donors, in steady state. Moreover, effects of a synthetic GC, triamcinolone acetonide (TA), on human hematopoietic $CD34^+$ progenitor cells have not yet been tested. The effects of GC were also investigated.

Previously, it has not been reported whether human hematopoietic stem cells express the MCR and respond to mineralocorticoids. Mineralotropic hormones regulate the hydrosodic balance in many cell types via ion channels in the cell membrane [26,27]. The presence of MCR has been demonstrated in several animal tissues including the brain [28,29], heart [30,31], and secretory glands [4,26,32]. The possible presence of the MCR in HSC seemed worth investigating because these cells are the target of steroids related to MC [25]. Here we report that MCR is indeed present and we reveal the effects of a specific antagonist of MCR, ZK91587, on the formation of colonies of $CD34^+$ cells.

MATERIALS AND METHODS

Cells

Normal peripheral blood cells (PBC) were obtained from adult volunteers after informed consent. Cells were separated on a Ficoll-Hypaque gradient (specific gravity: 1.077 g/ml) (Eurobio, Paris, France), washed twice with RPMI 1640 supplemented with 1% L-glutamine and 1% penicillin-streptomycin (Gibco, Cergy-Pontoise, France) and further depleted of adherent cells by incubation in 75 cm^2 plastic flasks (Costar) 2 hr in Iscove Modified Dulbecco's Medium (Gibco) containing 10% fetal calf serum (FCS; Dutscher, Brumath, France). HSC were obtained by positive selection of CD34 expressing cells (Myltenyi Biotec, Tebu, Le Perray-en-Yvelines, France). $CD34^+$ HSC are indirectly magnetically labeled by using a hapten-conjugated primary monoclonal antibody and an anti-hapten antibody coupled to microbeads. The magnetically labeled cells are enriched on positive selection columns in the magnetic field.

Reagents

Cytokines. Rh Epo was purchased from Boehringer (Mannheim, Germany) and rh IL-3 (2×10^7 UI/mg) from Tebu. RhG-CSF glycosylated (33.6×10^6 UI/263 μg) was a generous gift from Roger Bellon (Chugai-Rhône-Poulenc, France).

Steroids. Triamcinolone acetonide (TA), aldosterone (Aldo), deoxycorticosterone (DOC) (Sigma), the antimineralocorticoid ZK91587 (NEN products), and the high-affinity antiglucocorticoid RU38486 (Mifepristone, kindly provided by Dr. R. Sitruk-Ware, Laboratories Excelgyn, Paris, France), were dissolved in ethanol and stored at −20°C. They were diluted in culture medium immediately before use. Ethanol concentration in the cultures was 0.01%. In all experiments, controls were made with this concentration of ethanol. No measurable effect on colony formation was observed.

Serum-free Colony Assay

Human peripheral blood cells $CD34^+$ (5×10^3 cells/ml) separated as described above, were plated in 35-mm Petri dishes (Costar, Strasbourg, France) at a final volume of 1 ml per dish in Easy Mega™ serum-free collagen semi-solid medium (Hemeris, France), containing bovine serum albumin (1.5%), L-glutamine (2 mM), 2β mercapto-ethanol (100 μM), pyruvic acid (0.1 mM), $CaCl_2$ (250 μM), lipid mixture (4%), bovine pancreatic insulin (10 μg/ml), human transferrin (300 μg/ml) in IMDM solution, in presence of Epo, IL-3, and G-CSF. Cultures were incubated at 37°C in a 5% CO_2 humidified incubator for 14 days and scored for burst-forming unit-

erythroid (BFU-E) and colony-forming unit granulocyte-macrophage (CFU-GM) colonies. All growth factors were used at predetermined optimal concentrations: rhEpo, 20 UI/ml, rhIL-3, 20 UI/ml; rhG-CSF, 100 UI/ml.

Collagen Gel Dehydration

The collagen matrix of Easy-Mega permits an entire gel culture to be harvested on a glass slide. This process preserves, after dehydration, cellular morphology and colony structure. It also allows cytological staining (Hematoxylin-DAB staining) of erythroid colonies.

Hematoxylin-DAB Staining

This cytological stain was used to detect hemoglobin presence with a peroxydase reaction. As soon as the collagen was dry, slides were fixed in methylic alcohol for 10 min. Slides were then incubated with a mixture of H_2O_2, ethanol, diaminobenzidine (DAB) for 5 min and washed with tap water. After drying, slides were transferred to hematoxylin solution for 10 min, washed and dried again.

Immunocytochemistry

$CD34^+$ cells were fixed onto slides by cytospin. Slides were covered sequentially with anti-MCR antiserum (1:100), anti-human IgG biotinylated antibody (1:200), and streptavidin-FITC, all diluted in phosphate-buffered saline + 1% bovine serum albumin (BSA). Each incubation step (60 min at room temperature) was followed by extensive washing with phosphate-buffered saline and the slides were finally mounted in Fluoprep.

Statistical Methods

The results expressed as the mean ± SD were obtained for four or more separate experiments. Statistical significance was determined by using the Student's *t*-test.

Results

Characterization of Colony Formation From $CD34^+$ PBC

It is generally thought that serum contains colony-stimulating factors (CSFs) and other substances that are capable of modulating the effects of steroids such as GC or MC on both erythroid and GM colony formation. To rule out this possibility, we cultivated 5×10^3 $CD34^+$ cells per dish in serum-free collagen medium and tested effects of GC and MC with or without their antagonist. The serum-free collagen culture assay supplemented with Epo, IL-3, and G-CSF allows development of both CFU-GM and BFU-E in culture and here determination of the effects of additional factors on both CFU-GM and BFU-E. The influence of a synthetic GC (TA), a GC antagonist (RU38486), two MC (Aldo, DOC), and a MC antagonist (ZK91587) on colony formation was assessed by 14 day cultures. Results were expressed as % of ethanol control.

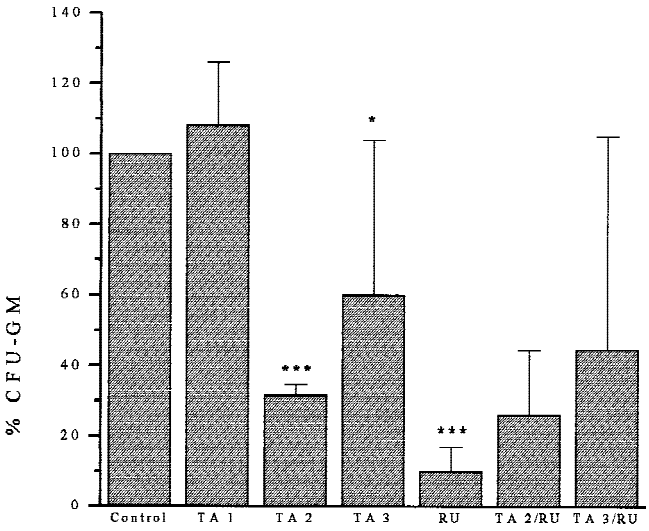

Fig. 1. Effect of TA and/or RU38486 on GM colony formation from $CD34^+$ PBC in serum-free collagen culture stimulated by IL-3, G-CSF, and Epo. The results are expressed as % obtained in six separate experiments ± SD of GM colonies per 5×10^3 cells. By convention, control (Ethanol) culture is assigned 100%. The Student's *t*-test was used to determine the level of statistical significance. TA1: 10^{-7} M, TA2: 10^{-6} M, TA3: 10^{-5} M, RU: 10^{-5} M. Difference in the number of GM colonies after treatment with TA 10^{-6} M or RU 10^{-5} M alone versus control was considered significant at $p < 0.001$ by Student's *t*-test. Difference in the number of GM colonies after treatment with TA 10^{-5} M versus control was considered significant at $p < 0.01$ by Student's *t*-test.

Effects on GM Colony Formation

Glucocorticoid. Our results show a significant decrease in GM-colony numbers in the presence of TA alone (10^{-6} and 10^{-5} M) and RU38486 alone (10^{-5} M), respectively 31.6 ± 3%, 60 ± 44% and 10 ± 7%. Moreover, RU38486 (10^{-5} M) failed to antagonize the TA effect (Fig. 1).

Mineralocorticoid. DOC was tested at two concentrations. At the concentration of 10^{-6} M, DOC alone significantly decreased GM colony formation (respectively 64 ± 58% and 20 ± 13%). In contrast, at a higher concentration, DOC (10^{-5} M) alone, and Aldo (10^{-5} M) alone significantly increased CFU-GM number (respectively 128.5 ± 50.5% and 156 ± 36%). Furthermore, ZK (10^{-5} M) antagonized the effects of DOC at the highest concentration (10^{-5} M). In contrast, ZK (10^{-5} M) failed to antagonize the effects of DOC (10^{-6} M) and Aldo (10^{-5} M) (Fig. 2). Alone, ZK, at the concentration of 10^{-5} M significantly decreased CFU-GM numbers.

Effects on Erythroid Colony Formation

Glucocorticoid. To investigate whether TA could affect the proliferation of human progenitors, 5×10^3

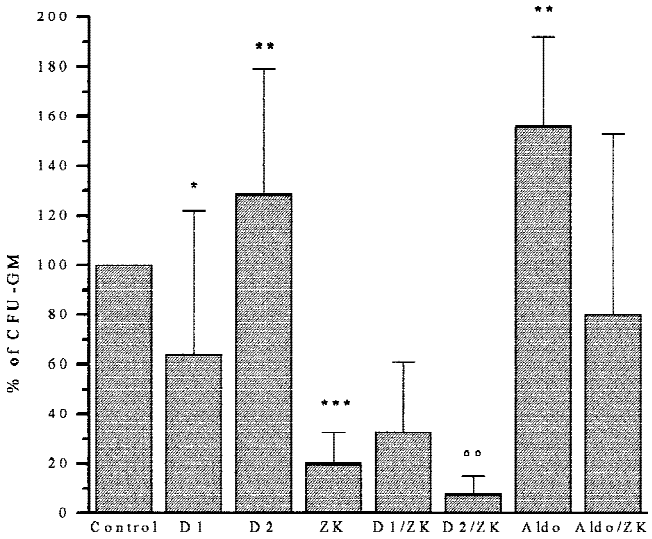

Fig. 2. Effect of DOC (D) or Aldo and/or ZK91587 on GM colony formation from CD34⁺ PBC in serum-free collagen culture stimulated by IL-3, G-CSF, and Epo. The results are expressed as % obtained in six separate experiments ± the standard deviation of GM colonies per 5 × 10^3 cells. By convention, control (Ethanol) culture is assigned 100%. The Student's *t*-test was used to determine the level of statistical significance. D1: 10^{-6} M, D2: 10^{-5} M, ZK: 10^{-5} M, Aldo: 10^{-5} M. *Differences in the number of GM colonies after treatment with DOC 10^{-6} M, DOC 10^{-5} M, Aldo 10^{-5} M or ZK 10^{-5} M alone versus control were considered significant at respectively $p < 0.01$, $p < 0.005$, $p < 0.005$, and $p < 0.001$ by Student's *t*-test. °Difference in the number of GM colonies after treatment with DOC 10^{-5} m and ZK 10^{-5} M versus DOC 10^{-5} M alone or versus ZK 10^{-5} M alone was considered significant at $p < 0.005$ by Student's *t*-test.

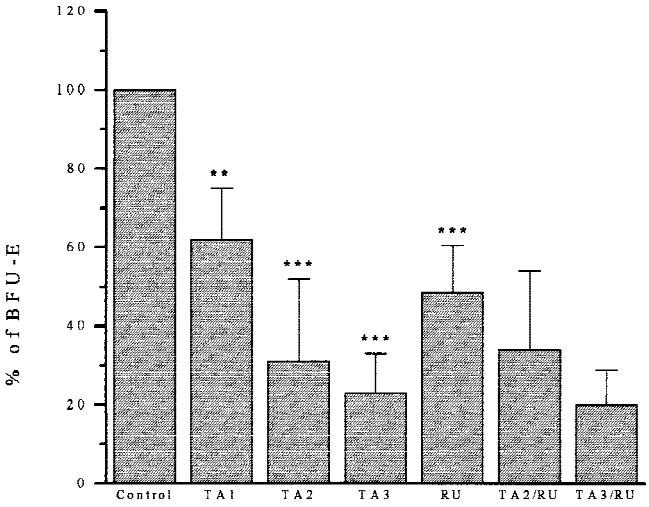

Fig. 3. Effect of TA and/or RU38486 on erythroid colony formation from CD34⁺ PBC in serum-free collagen culture stimulated by IL-3, G-CSF, and Epo. The results are expressed as % obtained in six separate experiments ± the standard deviation of erythroid colonies per 5 × 10^3 cells. By convention, control culture is assigned 100%. The Student's *t*-test was used to determine the level of statistical significance. TA1: 10^{-7} M, TA2: 10^{-6} M, TA3: 10^{-5} M, RU: 10^{-5} M. Difference in the number of BFU-E after treatment with TA 10^{-7} M versus control was considered at $p < 0.05$ by Student's *t*-test. Differences in the number of BFU-E after treatment with TA 10^{-6} M, TA 10^{-5} M, or RU 10^{-5} M versus control were considered significant at p < 0.001 by Student's *t*-test.

CD34⁺ cells were cultured in Easy Mega™ with Epo, IL-3, and G-CSF, in the presence of different concentrations of TA. Results of experiments are presented in Figure 3. TA (10^{-7}–10^{-5} M), significantly decreased the number of erythroid bursts (respectively 62 ± 13%, 31 ± 21%, 23 ± 10%). TA also strongly inhibited erythroid colony formation in a dose-dependent manner. RU38486 alone (10^{-5} M) significantly decreased erythroid colony formation (48.5 ± 12). Moreover, RU38486 (10^{-5} M) did not antagonize the inhibitory effects of TA (10^{-5} M; Fig. 3).

Mineralocorticoid. DOC (10^{-6} and 10^{-5} M), Aldo (10^{-5} M), ZK91587 (10^{-5} M) induce a significant decrease of erythroid colonies (respectively, 62 ± 16%, 41 ± 12%, 77 ± 22%, 54 ± 14%). The addition of ZK91587 (10^{-5} M) to DOC (10^{-5} M) resulted in a considerable decrease in number of erythroid colonies (13 ± 8%), that was significantly greater than the ones obtained with either ZK 91587 or DOC (10^{-5} M) alone suggesting a synergistic effect of the two compounds (Fig. 4).

Effects on the Morphology of Erythroid Bursts of CD34⁺ Cells

The effects of both GC and MC, in the absence or presence of their antagonist, on the size of erythroid bursts generated by CD34⁺ cells were investigated. Morphology of erythroid colonies are presented in Figures 5 and 6.

Glucocorticoid. TA (10^{-5} M) strongly affected the morphology of erythroid colonies supported by IL-3, G-CSF, EPO, and ethanol (Fig. 5). In the presence of TA, erythroid colonies are smaller than control colonies.

Mineralocorticoid. Neither DOC (10^{-5} M) nor ZK (10^{-5} M) alone affected the size of erythroid colonies. However, when ZK (10^{-5} M) was added to DOC (10^{-5} M), the size of BFU-E derived colonies was markedly diminished (Fig. 6).

Effects on the Maturation of Erythroid Bursts from CD34⁺ PBC

Finally, we tested the effects of GC and MC on erythroid maturation.

Glucocorticoid (Fig. 7). Erythroid maturation due to the presence of hemoglobin was evaluated by the peroxydasic activity. No significant modification of erythroid maturation was observed in the presence of RU (10^{-5} M) compared with the control culture (ethanol). In contrast, an enhancement of erythroid maturation, with a high level of hemoglobin, was observed in the presence of TA (10^{-5} M). TA induced both nuclear and cytoplasmic differentiation. Interestingly, in the presence of both

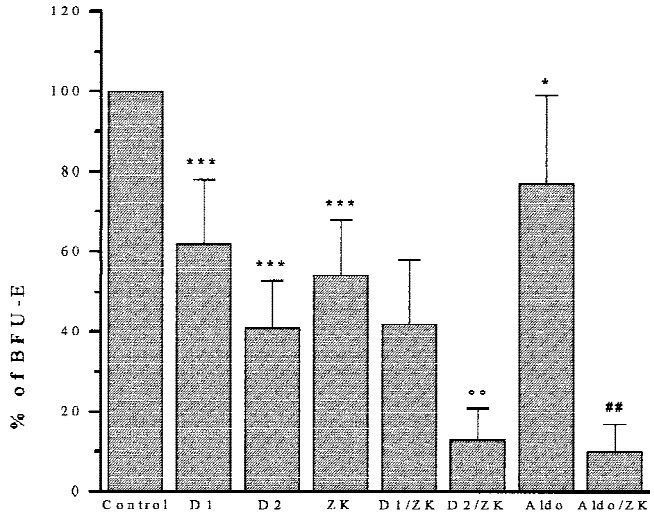

Fig. 4. Effect of Aldo or DOC and/or ZK91587 on erythroid colony formation from CD34$^+$ PBC in serum-free collagen culture stimulated by IL-3, G-CSF, and Epo. The results are expressed as % obtained in six separate experiments ± the standard deviation, or erythroid colonies per 5 × 10^3 cells. By convention, control (ethanol) culture is assigned 100%. The Student's *t*-test was used to determine the level of statistical significance. D1: 10^{-6} M, S2: 10^{-5} M, ZK: 10^{-5} M, Aldo: 10^{-5} M. *Differences in the number of BFU-E after treatment with DOC 10^{-6} M, DOC 10^{-5} M, or ZK 10^{-5} M alone versus control were considered significant at $p < 0.001$ by Student's *t*-test. Difference in the number of BFU-E after treatment with Aldo 10^{-5} M alone versus control were considered significant at $p < 0.01$ by Student's *t*-test. °Difference in the number of BFU-E after treatment with DOC 10^{-5} M and ZK 10^{-5} M versus DOC 10^{-5} M alone or versus ZK 10^{-5} M alone was considered significant at $p < 0.005$ by Student's *t*-test. #Difference in the number of BFU-E after treatment with Aldo 10^{-5} M and ZK 10^{-5} M versus Aldo 10^{-5} M alone or versus ZK 10^{-5} M alone was considered significant at $p < 0.005$ by Student's *t*-test.

RU (10^{-5} M) and TA (10^{-5} M), a decreased peroxydasic activity was observed, indicating the presence of few intracytoplasmic hemoglobin. Moreover, the size of nuclear was greater and chromatin appeared less compact.

Mineralocorticoid (Fig. 8). No modification of erythroid maturation was observed in the presence of DOC (10^{-5} M) and/or ZK (10^{-5} M).

Immunocytochemistry

Immunocytochemical analysis with an antibody to human MCR [31,44] confirmed that this receptor is indeed expressed in human CD34$^+$ stem cells (Fig. 9). Most HSC CD34$^+$ presented a positive cytoplasmic staining for the MCR. The cytoplasm of most of the CD34$^+$ cells stained positively for the MCR.

DISCUSSION

Both stimulatory and inhibitory factors may contribute to hematopoietic regulation. Expansion and maturation of immature progenitor cells appear to be linked to the control of proliferation and apoptosis [33]. Proliferation of HSC in vitro is regulated by many types of cytokines and by the microenvironment of stromal cell layers. Although there is good evidence that steroid hormones affect many stages of erythropoiesis, their precise mechanism of action remains unclear [34–36]. It is possible that they enhance the responsiveness of red cell precursors to erythropoietin or alternatively that they directly stimulate cell proliferation [22]. Golde et al. (1976) reported that the GC dexamethasone potentiates the Epo-dependent proliferation of red cell progenitors in vitro [19]. The dexamethasone effect may involve an increased sensitivity of progenitors cells to erythropoietin. Recently, Wessely et al. demonstrated that the GCR is essential for the long-term proliferation of avian erythroid progenitors [37–39]. A chemokine, MIP-1α, induces inhibition of hematopoietic progenitors. Chikkappa et al. hypothesized that the hematopoiesis promoting effect of GC may be by inhibition of MIP-1α production from accessory cells [40]. However, B. Cylwick et al. found that GC inhibit the GM colony formation in vitro [18]. Our results demonstrate a direct inhibition by triamcinolone acetonide, a GC, of both erythroid and GM colony formation from human CD34$^+$ PBC. Furthermore, TA affects markedly the morphology of erythroid colonies. The transcriptional response of CD34$^+$ cells to GC is blocked by a potent antagonist like RU38486 (Mifepristone), which has proved to be a remarkably antiglucocorticosteroid agent in humans. One mechanism of action of RU38486 involves the intracellular receptor of GC [41]. However, treatment of human CD34$^+$ cells, in vitro, with a high concentration of RU38486 did not reverse the inhibitory effect of TA suggesting that the inhibitory effect of GC is mediated by different mechanisms from those described by Wessely et al.

In the present study, we provide singular evidences based on antibody cross-reactivity and colony formation and morphology for the presence of receptors for MC in human PBSC. In accord with previous studies, human MCR was detected principally in the cytoplasmic compartment of PB CD34$^+$ cells [42]. The fact that HSC responded to aldosterone suggests that the MCR is functional. Aldosterone increased GM colony formation. The opposite effects of aldosterone on erythroid colony formation suggests that this mineralotropic hormone may act as a growth regulatory agent on cells of the hematopoietic lineage. These data are consistent with the regulation of HSC proliferation and differentiation by other steroid hormones acting via their specific receptors in HSC [25]. Synthetic spirolactone (ZK91587) binds specifically to the peripheral MCR [4,5,32] and reverses specifically the MC effects [43,44]. Here, we report that both the stimulatory and inhibitory effects of aldosterone (10^{-5} M) were not blocked by ZK91587. In contrast, the

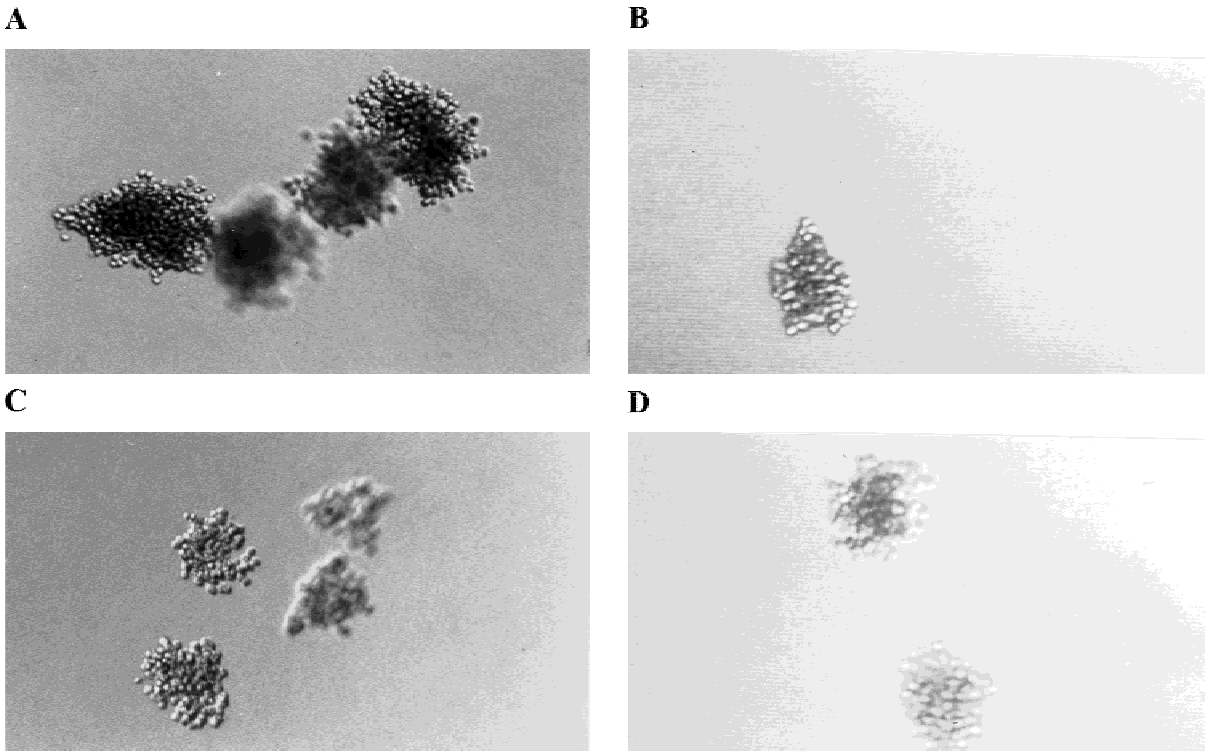

Fig. 5. Photography of an erythroid colony generated when CD34$^+$ PBC are cultured in serum-free collagen medium with IL-3, G-CSF, and EPO. (A) Control (Ethanol), (B) TA (10^{-5} M), (C) RU (10^{-5} M), (D) TA/RU.

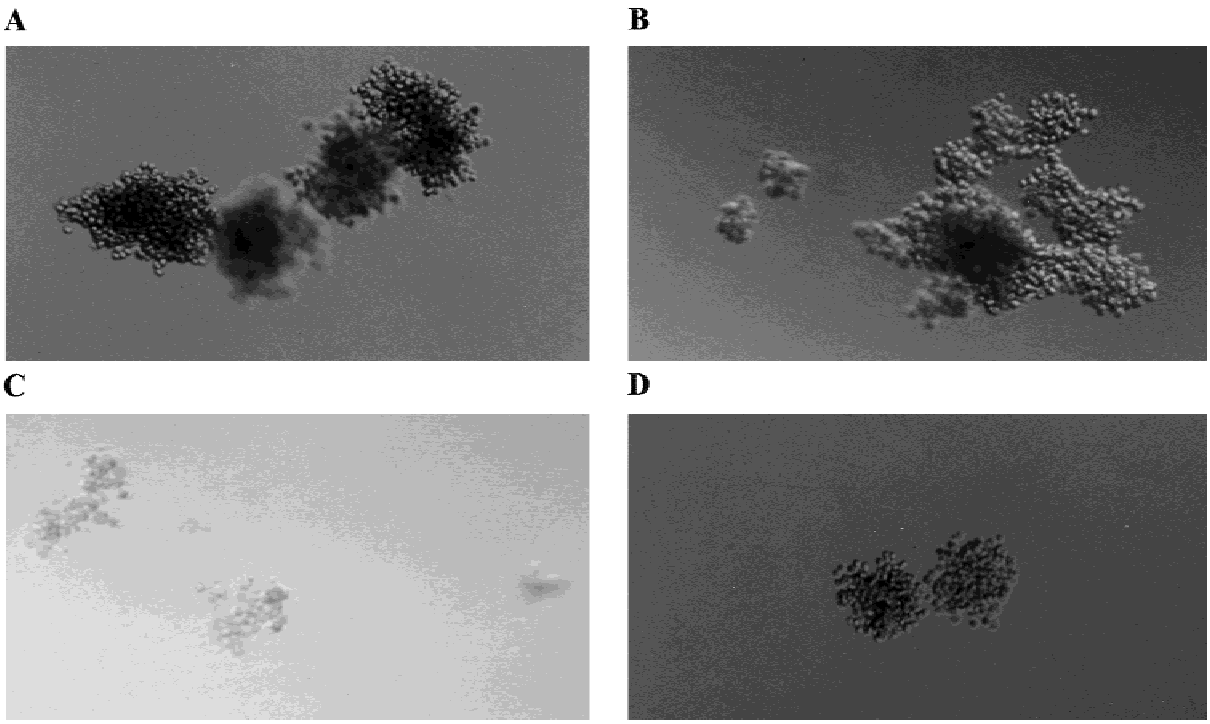

Fig. 6. Photography of an erythroid colony generated when CD34$^+$ PBC are cultured in serum-free collagen medium with IL-3, G-CSF, and EPO. (A) Control (Ethanol), (B) DOC (10^{-5} M), (C) ZK (10^{-5} M), (D) DOC/ZK.

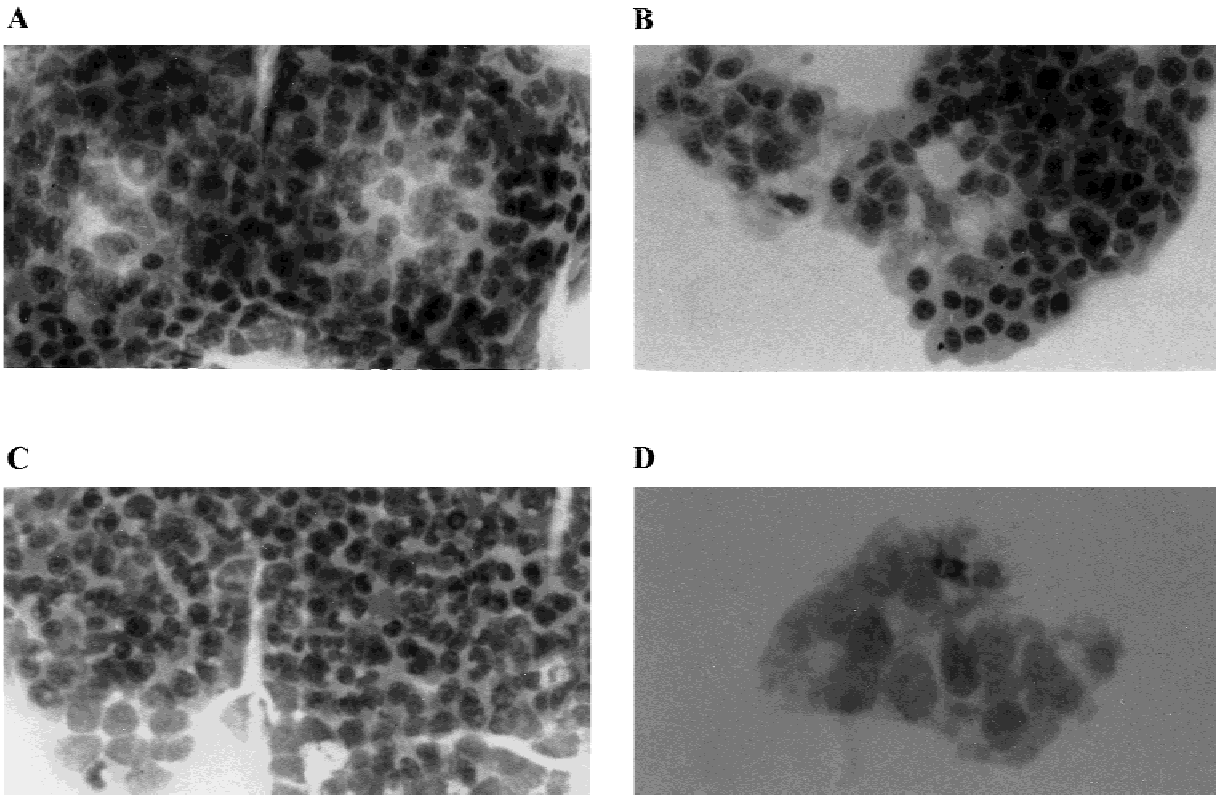

Fig. 7. Photography of an erythroid colony derived from CD34$^+$ PBC, in serum-free collagen medium, in the presence of IL-3, G-CSF, and EPO, on hematoxylin-DAB staining. (A) Control (Ethanol), (B) TA (10^{-5} M), (C) RU (10^{-5} M), (D) TA/RU.

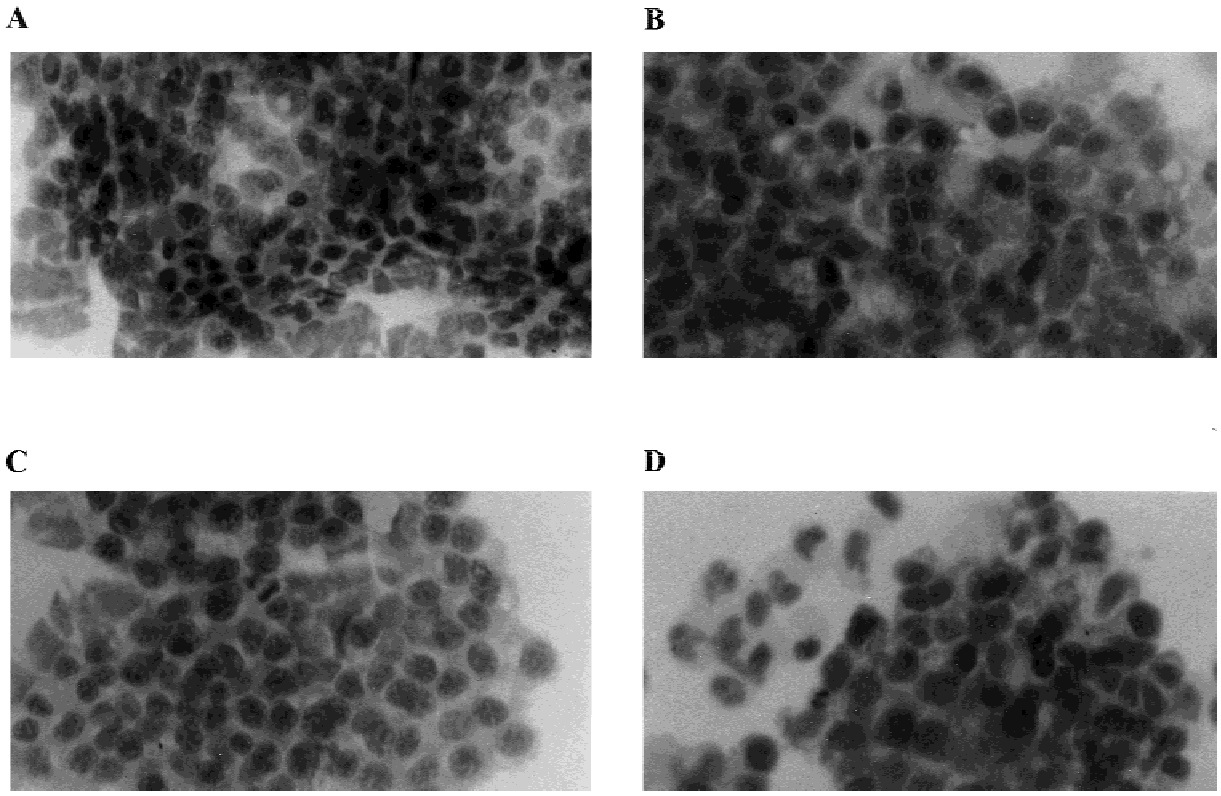

Fig. 8. Photography of an erythroid colony derived from CD34$^+$ PBC, in serum-free collagen medium, in the presence of IL-3, G-CSF, and EPO, on hematoxylin-DAB staining. (A) Control (Ethanol), (B) DOC (10^{-5} M), (C) ZK (10^{-5} M), (D) DOC/ZK.

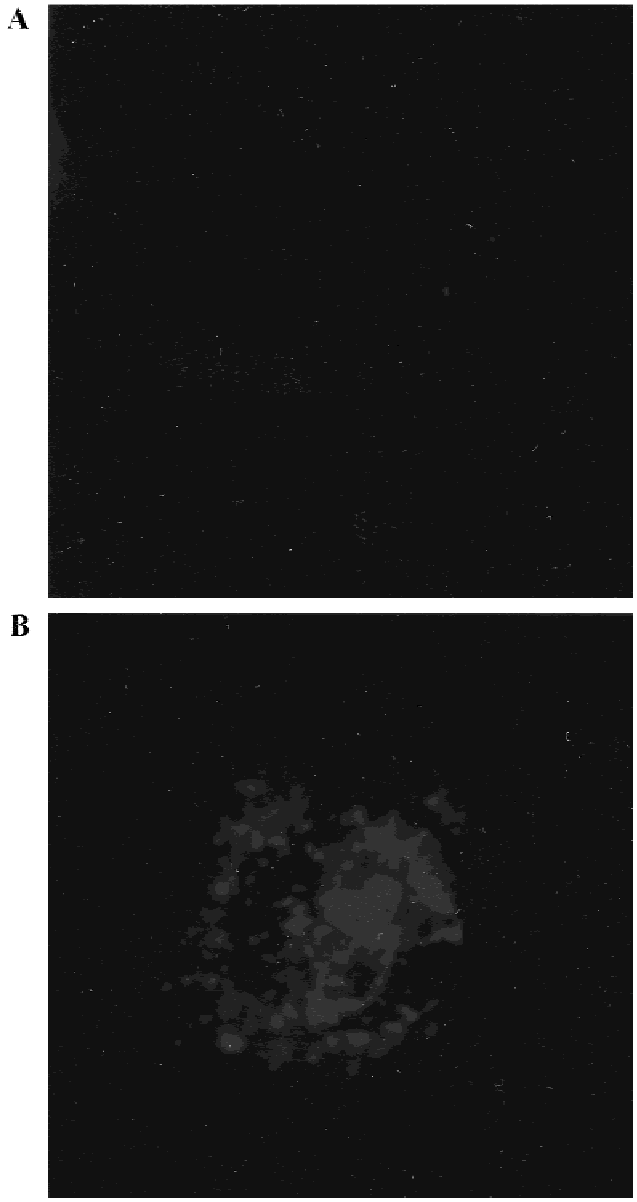

Fig. 9. Immunocytochemical localization of MCR in CD34+ PBC. Cells on slides were fixed and incubated with 1:100 dilution of an antiserum specifically directed against MCR (B) or with a nonimmune serum (A). Most CD34+ cells showed a positive staining for MCR that appear perinuclear.

stimulatory effect of DOC (10^{-5} M) on CFU-GM was reversed by ZK91587. These data extend earlier studies suggesting that aldosterone mediates some of its physiological effects via binding with relatively low affinity to GCR, despite the presence of apparently normal MCR [45].

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